IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:

Applicants	:	Hansen et al.	
Appln. No.	:	10/561,823	Confirmation No: 5908
Filed	:	December 19, 2005	Group Art Unit: 1636
Title	:	METHOD OF PRODUCING A LOW MOLECULAR WEIGHT ORGANIC COMPOUND IN A CELL	Examiner: Michele K. Joike

DECLARATION UNDER 37 CODE OF FEDERAL REGULATIONS § 1.132

DECLARATION OF PROFESSOR BIRGER LINDBERG MØLLER

Commissioner for Patents
P.O. Box 1450
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City of Copenhagen

Country of Denmark

To the Commissioner:

I, Professor Birger Lindberg Møller, being duly sworn, depose and say:

1. I am an owner of the above-identified application and a person of ordinary skill in the art in the field of the subject matter of the above-captioned application for patent. I am also completely familiar with the contents of the above-captioned patent application and also with the disclosures contained in Moehs et al. (The Plant J. 11(2): 227-236, 1997, hereinafter "Moehs"), Day et al. (FEBS letters 486 (1998); hereinafter "Day"), Arend et al. (Biotechnol. Bioeng. 76(2):126-31, 2001, esp. pp. 129-130; hereinafter "Arend") and Priefert (Applied Microbiol. Biotechnol. 56:296-314 (2001); hereinafter "Priefert"), cited in the Office Action of November 5, 2009, in connection with the above-identified patent application and in which certain claims were rejected over the disclosures of these references.

2. I am a professor of plant biochemistry and Head of the research centre Pro-Active Plants founded by the Villum Kann Rasmussen Foundation and Deputy Director of the Synthetic Biology research centre, one of four "centres of excellence" in Denmark established by the Ministry of Science, Technology and Innovation in 2009. I graduated from the University of Copenhagen, Denmark in 1972, receiving a MSc degree in plant biochemistry and organic chemistry; I subsequently obtained my PhD from the University of Copenhagen, Denmark in 1972, and received my DSc degree in plant biochemistry from the University of Copenhagen,

Denmark, in 1984. I was employed from 1975 to 1977 as a Fulbright scholar in plant biochemistry at the University of California, Davis, USA. From 1977 to 1984 I was employed as Senior Research Scientist and Niels Bohr Fellow of the Royal Danish Academy of Sciences at the Physiology Department of the Carlsberg Laboratory. In 1984, I was appointed as Research Professor at the Royal Veterinary and Agricultural University (now faculty at the University of Copenhagen). In 1989, I was appointed Full Professor in Plant Biochemistry at the University of Copenhagen, a position I am still holding. In the period 1998-2008 I have been Head of Center for Molecular Plant Physiology, the center of excellence in plant biology in Denmark established by the National Danish Research Foundation. In 1998, I was appointed Head of a new center of excellence supported by a grant from the Villum Kann Rasmussen Foundation. In 2009, I was appointed Deputy Director of a new research centre within Synthetic Biology at the University of Copenhagen established by the Ministry of Science, Technology and Innovation as one of four centres in Denmark. From 1992 to this date I have been a member of the Danish Board of Patent Appeals appointed by the Danish Minister of Finance. Throughout my career, my key research interest has been the biochemistry of bioactive natural products and the cytochromes P450 and glycosyltransferases involved in their biosynthesis. This work includes designing and establishing a synthetic pathway for vanillin and vanillin glucoside in yeasts (J. Envir. Appl. Microbiol 75: 2765-2774 (2009)) where I am engaged in the challenge to identify a glucosyltransferase able to convert vanillin into the corresponding glucoside and in screening yeast strains for their ability to produce vanillin and vanillin glucoside.

3. Therefore, being completely familiar with the subject matter of the patent application including both the specification and claims, the cited references, and the properties of the methods in the patent application and in the references, it is clearly evident to me that, on comparison of the latter methods of the cited art with those of the claims in the present application, the claims in the present application present unobvious and patentable advances over the cited art, particularly because of the superior, unobvious, un-expected, and different properties of the methods and compounds of the instant specification and claims, as is set forth below.

4. As is set forth in the specification of the present application, at para. [0017] – [0020] (publication version US 2006/0275877), the present application compares a microorganism into which has been inserted genes involved in the biosynthesis pathway leading to a low molecular weight aglycon compound with the same microorganism into which in addition has been introduced a glycosyltransferase gene capable of glycosylating the produced aglycon and as a result obtain within the microorganism the corresponding glycosylated form of the aglycon. The findings presented in this portion of the specification set forth that the microorganism with the glycosyltransferase during culture fermentation is capable of producing higher amounts of the glycosylated form of the aglycon as compared to the amounts of the corresponding aglycon produced by the microorganism without the glycosyltransferase. The state of the cited art fails to illustrate, teach, suggest, or motivate this development. The "overproduction" of the compounds of the instant development and the absence of this activity in the prior art compound is unexpected and unobvious.

5. As set forth in the specification of the present application, para. [0014] Moehs, C P et al, Plant Journal (1997) 11:227-236 merely describes that a cDNA encoding a solanidine glucosyltransferase (SGT) was isolated from potato. The cDNA was selected from a yeast expression library using a positive selection screen based on the higher toxicity of steroidal alkaloid aglycons relative to their corresponding glycosylated forms. The activity of the expressed cloned SGT was tested in an **in vitro** assay based on isolated recombinant produced SGT. The application of the cloned SGT brought forward by Moehs is clearly summarized in the last sentence of the introduction: "The molecular cloning of SGT opens the possibility of developing novel methods to decrease SGA levels in potato cultivars by down-regulating the expression of this enzyme using antisense RNA transgenes" (end of citation). Moehs thus advises to downregulate SGT expression to reduce the level of the accumulated glucoside.

This advice is in direct opposition to the approach of the instant application which teaches introduction of genes encoding enzymes of a biosynthetic pathway for an aglycon concomitant with introduction of a glucosyltransferase to substantially convert the aglycon formed into the corresponding non-toxic glucoside with the aim of increasing the level of a desired glucoside. The promoter used to drive the expression of the glucosyltransferase encoding gene is strong and provides quick conversion of the aglycon into the corresponding non-toxic glucoside. It is my clear opinion that Moehs serves to highlight in a very clear manner that the technologies described in the present application possess a number of properties entirely different from and not shown or indicated by Moehs or the other cited art.

6. Moehs teaches that a glycosylated form of solasodine may be obtained when the relevant glycosyltransferase (SGT, solanidine glucosyltransferase) is incubated **in vitro** with the isolated recombinant SGT in the presence of UDPG and solasodine. However, Moehs does not at all teach or suggest whether the yeast or E. coli could produce the glycosylated form of the solasodine **in vivo** because no relevant biosynthesis pathway genes for solasodine were introduced in the described yeast or E. coli cells. The formation of the glycoside as reported in Moehs occurs outside the cell. The SGT enzyme is isolated from the cells expressing SGT (solanidine glucosyltransferase), and this enzyme is then used in test tube experiments to glycosylate exogenously added solasodine. Thus it is not shown that the glucosylated compound may be obtained under **in vivo** conditions of the cell where enzyme and substrate compartmentalization or unfavorable pH values may obstruct product formation. In the instant application we demonstrate that the aglycon may be efficiently glucosylated **in vivo** without accumulation of the aglycon, the aglycon perhaps being toxic. Likewise, industrial production of vanillin glucoside according to the methodology of Moehs is not economically feasible because the process requires addition of stoichiometric amounts of highly expensive UDPG (the activated glucose donor used by the enzyme). In some examples of the present patent application, live yeast cells are producing the required amounts of UDPG for vanillin glucoside production themselves, i.e., the technology platform presented in the present patent application renders vanillin glucoside production in yeast of economical interest. This again serves to highlight the differences between previous art and the technology platforms presented in the present application.

7. Moehs also does not teach, suggest or motivate the possibility of obtaining increased amounts of the desired aglycon by first forming large amounts of the glucosylated aglycon and then liberating the free aglycon again by treatment with a deglycosylating agent such as beta-glucosidase. The purpose for the glycosylation in Moehs is to render the externally added "toxic" solasodine (the aglycon) less harmful so that the yeast cell will grow faster in an environment into which solasodine is administered from an external source. Indeed, for this reason, deglycosylation is contra-indicated as it would inhibit as opposed to enhance yeast cell growth. As outlined above (under paragraph 6), in the present application, the technology includes substantially simultaneous expression of the genes encoding enzymes responsible for the synthesis of the aglycon and of the glucosyltransferase converting the aglycon into the corresponding glucoside. This avoids accumulation of the toxic aglycon. Using the technology outlined in the instant application, growth retardation of the yeast cells would not be encountered because the toxic aglycon (solasodine) would not accumulate. Thus the problem that Moehs proposes to solve is not encountered using the technology described in the present application and claims.

8. Figure 7 of Moehs is not relevant to the present application because the data reported in Figure 7 represent the ***in vitro***-tested activity of the cloned enzyme SGT. For the testing of Figure 7, the recombinantly produced solanidine glucosyltransferase SGT is tested for the desired activity ***in vitro***. It is clear that the amount of glucoside produced is higher in the experiments where enzyme extracts from yeast expressing the SGT gene were used in comparison to extracts of yeast that did not contain the SGT encoding gene. However, these experiments are carried out **in vitro** and involve addition of the aglycon and demonstration of its conversion into the corresponding glucoside when the yeast extract is made from a yeast

expressing the glucosyltransferase. It is clear that no glucoside would be formed using a yeast extract not expressing the glucosyltransferase encoding gene. These results cannot therefore be compared to the overproduction of the glucoside reported in the present application where the yeast harbors the genes encoding the enzymes required for the synthesis of the aglycon as well as the glucosyltransferase. In the present application, it may be that the overproduction is achieved because the aglycon is toxic to the living yeast cells and this toxic effect is relieved by converting the toxic aglycon into the nontoxic corresponding glucoside. The results presented in Figure 7 of Moehs are therefore not comparable to the results on overproduction achieved in the present application. Moehs does not teach, suggest or motivate the combination of operations of the developments presented in the claims of the present application.

9. The conclusions of the Office Action of November 5, 2009, page 4, lines 10-11 and 14-15, that "[b]oth the SGT and the solanidine genes were introduced into *S. cerevisiae*" and that "[Moehs] also shows that the cell is capable of producing higher amounts of glycosylated solanidine with SGT present, than without" are inaccurate or at the very least mis-leading in the present context. As described in paragraphs 6, 7 and 8 hereabove, the cells of Moehs did not have introduced therein both the genes for production of the aglycon solasodine as well as the genes for the production of the enzyme SGT. As the conclusions of the Office Action of November 5, 2009, page 4, lines 10-11 and 14-15 are therefore based on inaccurate assumptions and present an inaccurate summarization of the Moehs process, the presently-claimed subject matter is not obvious in view thereof.

10. Neither of the Day or Priefert cited references supplement these failures of Moehs to demonstrate or suggest the production in the cell of the glycosylated form of the aglycon. Indeed, neither Day nor Priefert were cited for such. Thus, no matter what Day may teach of deglycosylation of flavanoid, and/or no matter what Priefert may teach of the production of aglycon vanillin; neither teach or suggest or motivate one of skill in the art to cure the failures of Moehs; i.e., neither of Day nor Priefert, nor any combination thereof suggests or motivates the introduction into the yeast cell of genes for production of the aglycon as well as introduction into the yeast cell of genes for production of the glycosyltransferase for production of the glycosylated form of the aglycon.

11. As further set forth in the specification of the present application, para. [0013], Arend, J et al., Biotech. & Bioeng (2001) 78:126-131 and WO01/07631 merely describes cloning of a glucosyltransferase from the plant Rauvolfia serpentina. The cloned glucosyltransferase was inserted into E. coli bacteria. When the aglucones hydroquinone, vanillin and p-hydroxyacetophenone were added to the medium of cultivated cells of the engineered E. coli, the corresponding glucosides, arbutin, vanillin-D-glucoside and picein were synthesized.

12. As was the case with the Moehs disclosures, the Arend process involves merely the production within/by the E.coli of the glucosyltransferase enzyme, but not of the aglycon itself. Rather, the aglycon, e.g., vanillin here, is added to the medium wherein the glucosyltransferase then interacts therewith to achieve the glycosylation thereof. Arend does not cure the failure of Moehs to teach, suggest or motivate the introduction of discrete genes for production of both the aglycon and for the glucosyltransferase.

13. In contradistinction each instance of the examples from the cited art, and any combinations thereof, the claims and specification of the present application demonstrate differences that are novel and nonobvious over the state of the art. The processes of the present developments were found to be significantly more productive, and unexpectedly so, over the known methods in the art. Surprisingly, the present development's in-cell synthesis of both vanillin and the glycosylated version of the vanillin has been proven to improve the ultimate yield of vanillin upon deglycosylation of the glycosylated product.

14. Scientific acknowledgment of the achievements demonstrated by the present application can be found in the published article in Applied and Environmental Microbiology; de Novo Biosynthesis of Vanillin in Fission Yeast (*Schizosaccharomyces pombe*) and Baker's Yeast (*Saccharomyces cerevisiae*), Esben H. Hansen et al., APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2009, p. 2765–2774 (Exhibit A, attached hereto). This article corresponds to and reports on the developments of the present application and notes that vanillin is one of the world's most important flavor compounds, with a global market of $180 million. In the article, the authors, of whom I am one, establish a true de novo biosynthetic pathway for vanillin production from glucose in *Schizosaccharomyces pombe*, also known as fission yeast or African beer yeast, as well as in baker's yeast, *Saccharomyces cerevisiae*. The article illustrates that productivities were 65 and 45 mg/liter, after introduction of three and four heterologous genes, respectively. These de novo pathways represent the first examples of one-cell microbial generation of these valuable compounds from glucose. *S. pombe* yeast has not previously been metabolically engineered to produce any valuable, industrially scalable, white biotech commodity. In sum, in this peer-reviewed article, the method is shown to be the first of its kind and a nonobvious development over the prior art.

15. The present patent application was filed in June 2004. We published the data presented in the patent application in 2009 (see Exhibit A, described in paragraph 14, supra). In spite of this five year gap, the publication of the methods described in the subject application immediately garnered substantial attention in the scientific community. For example, the top ranked journal Nature Reviews in its Microbiology reports (Nature Reviews, Vol. 7, May 2009 (Exhibit B, attached hereto)) chose our paper as a top story and commented that

> "Hansen and colleagues have now produced strains of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* that can produce vanillin. They first searched for strains that did not convert vanillin to vanillyl alcohol. They then added genes from the dung mould *Podosporo pauciseta*, a bacterium of the *Nocardia* genus, and humans, which allowed the yeast strains to produce vanillin (an additional gene from *Corynebacterium glutamicum* was added to *S. cerevisiae* to activate the *Nocardia* enzyme). At 45–65 mg per litre, vanillin production was at a sufficient level to scale up for large-scale industrial production. These *de novo* pathways for vanillin synthesis in yeast represent the first examples of one-cell microbial generation of these valuable compounds from glucose."

Thus, the review article illustrates that the scientific community, which by definition are and/or include persons skilled in the art, understand this method to be novel and nonobvious.

16. Science News also selected our paper on vanillin glucoside production in yeast for commenting and discusses how the inventors increased the yeast yield of vanillin in this process by adding an additional gene that encodes for an enzyme that converts vanillin into its glycosylated form. The article explains that the glycosylated form is not toxic to the yeast, "allowing the yeast to hold more of the compound". Yeast Bred to Bear Artificial Vanilla, Rachel Ehrenberg, Science News, May 23, 2009, Vol. 175, No. 11, p. 9 (Exhibit C, attached hereto). The Science News journal contacted John Rosazza at University of Iowa who stated: "This is absolutely beautiful work." After having described how **de novo** synthesis of vanillin from glucose was achieved by insertion of four biosynthetic genes in two different yeast strains, the Science News journal also comments on the overproduction issue. The Science News article stated: "To further increase the yeast yield of vanillin, the researchers added an additional gene that encodes for an enzyme that converts the straight vanillin into a form with a sugar attached, vanillin-beta-D-glucoside. This form isn't toxic, says Møller, allowing the yeast to hold more of the compound."

17. In considering this data, it is clear that the art recognizes the developments hereof and that nothing in the cited art or in Moehs et al. would motivate one of ordinary skill in the art to use the specific approach set out by Applicants to achieve the production of high levels of vanillin glucoside by living yeast cells. First of all it is certainly not trivial to an ordinarily skilled artisan to identify all genes required for synthesis of a desired aglycon. Then these genes have to be heterologously expressed in a microbe like yeast and the enzymes need to be functionally active. Then a glycosyltransferase able to convert the aglycon into the non-toxic glucoside needs to be identified and expressed in a functional form and in a manner that enables it to convert the aglycon into the glucosylated product. Moreover, and though not necessary, it is not trivial or obvious to find a way to avoid the potentially toxic effects of the aglycon and to obtain the glucoside in amounts superior to those achieved by expression of the genes encoding for synthesis of the aglycon alone. Then a beta-glucosidase enabling the re-conversion of the glucoside into the desired free aglycon would need to be identified. All steps in this platform are technologically challenging for a person ordinarily skilled in the art. The achieved overproduction of vanillin glucoside and its easy, non-costly conversion into vanillin is highly remarkable because it represents a unique combination of a series of complex technologies. It could not be foreseen that the glucosyltransferase would work so well in yeast that it would offer the possibility to convert the aglycon into the glucosylated product within the environment within, e.g., at the pH value existing in the yeast cell and in spite of possible different localization and compartmentalization of the enzyme and its substrate. An ordinary skilled artisan would therefore not, based on the existing knowledge, be encouraged to embark in developing the combination of technologies necessary and presented in the present patent application and claims especially because the resulting overproduction could not have been foreseen to happen. Thus the economic incentive to develop the technology presented in the patent application could also not be envisioned.

18. For convenience, .pdf files of the published results and the developed technology platform of the present application and claims and the comments thereon in Nature Reviews and Science News are provided; Exhibits A, B and C, respectively.

19. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of the application, and any patent issuing thereon.

Date: December 30th, 2009

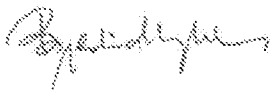

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EXHIBIT A

Applied and Environmental Microbiology, May 2009, p. 2765–2774 Vol. 75, No. 9
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De Novo Biosynthesis of Vanillin in Fission Yeast (*Schizosaccharomyces pombe*) and Baker's Yeast (*Saccharomyces cerevisiae*)▽

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Vanillin is one of the world's most important flavor compounds, with a global market of 180 million dollars. Natural vanillin is derived from the cured seed pods of the vanilla orchid (*Vanilla planifolia*), but most of the world's vanillin is synthesized from petrochemicals or wood pulp lignins. We have established a true de novo biosynthetic pathway for vanillin production from glucose in *Schizosaccharomyces pombe*, also known as fission yeast or African beer yeast, as well as in baker's yeast, *Saccharomyces cerevisiae*. Productivities were 65 and 45 mg/liter, after introduction of three and four heterologous genes, respectively. The engineered pathways involve incorporation of 3-dehydroshikimate dehydratase from the dung mold *Podospora pauciseta*, an aromatic carboxylic acid reductase (ACAR) from a bacterium of the *Nocardia* genus, and an *O*-methyltransferase from *Homo sapiens*. In *S. cerevisiae*, the ACAR enzyme required activation by phosphopantetheinylation, and this was achieved by coexpression of a *Corynebacterium glutamicum* phosphopantetheinyl transferase. Prevention of reduction of vanillin to vanillyl alcohol was achieved by knockout of the host alcohol dehydrogenase *ADH6*. In *S. pombe*, the biosynthesis was further improved by introduction of an *Arabidopsis thaliana* family 1 UDP-glycosyltransferase, converting vanillin into vanillin β-D-glucoside, which is not toxic to the yeast cells and thus may be accumulated in larger amounts. These de novo pathways represent the first examples of one-cell microbial generation of these valuable compounds from glucose. *S. pombe* yeast has not previously been metabolically engineered to produce any valuable, industrially scalable, white biotech commodity.

In 2007, the global market for flavor and fragrance compounds was an impressive $20 billion, with an annual growth of 11 to 12%. The isolation and naming of vanillin (3-methoxy-4-hydroxybenzaldehyde) as the main component of vanilla flavor in 1859 (6), and the ensuing chemical synthesis in 1874 (41), in many ways marked the true birth of this industry, and this compound remains the global leader in aroma compounds. The original source of vanillin is the seed pod of the vanilla orchid (*Vanilla planifolia*), which was grown by the Aztecs in Mexico and brought to Europe by the Spaniards in 1520. Production of natural vanillin from the vanilla pod is a laborious and slow process, which requires hand pollination of the flowers and a 3- to 6-month curing process of the harvested green vanilla pods (37). Production of 1 kg of vanillin requires approximately 500 kg of vanilla pods, corresponding to the pollination of approximately 40,000 flowers. Today, only about 0.25% (40 tons out of 16,000) of vanillin sold annually originates from vanilla pods, while most of the remainder is synthesized chemically from lignin or fossil hydrocarbons, in particular guaiacol. Synthetically produced vanillin is sold for approximately $15 per kg, compared to prices of $1,200 to $4,000 per kg for natural vanillin (46).

An attractive alternative is bioconversion or de novo biosynthesis of vanillin; for example, vanillin produced by microbial conversion of the plant constituent ferulic acid is marketed at $700 per kilogram under the trade name Rhovanil Natural (produced by Rhodia Organics). Ferulic acid and eugenol are the most attractive plant secondary metabolites amenable for bioconversion into vanillin, since they can be produced at relatively low costs: around $5 per kilogram (37). For the bioconversion of eugenol or ferulic acid into vanillin, several microbial species have been tested, including gram-negative bacteria of the *Pseudomonas* genus, actinomycetes of the genera *Amycolatopsis* and *Streptomyces*, and the basidiomycete fungus *Pycnoporus cinnabarinus* (19, 23, 25, 27, 31, 34, 35, 36, 45, 48). In experiments where the vanillin produced was absorbed on resins, *Streptomyces* cultures afforded very high vanillin yields (up to 19.2 g/liter) and conversion rates as high as 55% were obtained (15). Genes for the responsible enzymes from some of these organisms were isolated and expressed in *Escherichia coli*, and up to 2.9 g/liter of vanillin were obtained by conversion of eugenol or ferulic acid (1, 3, 32, 49).

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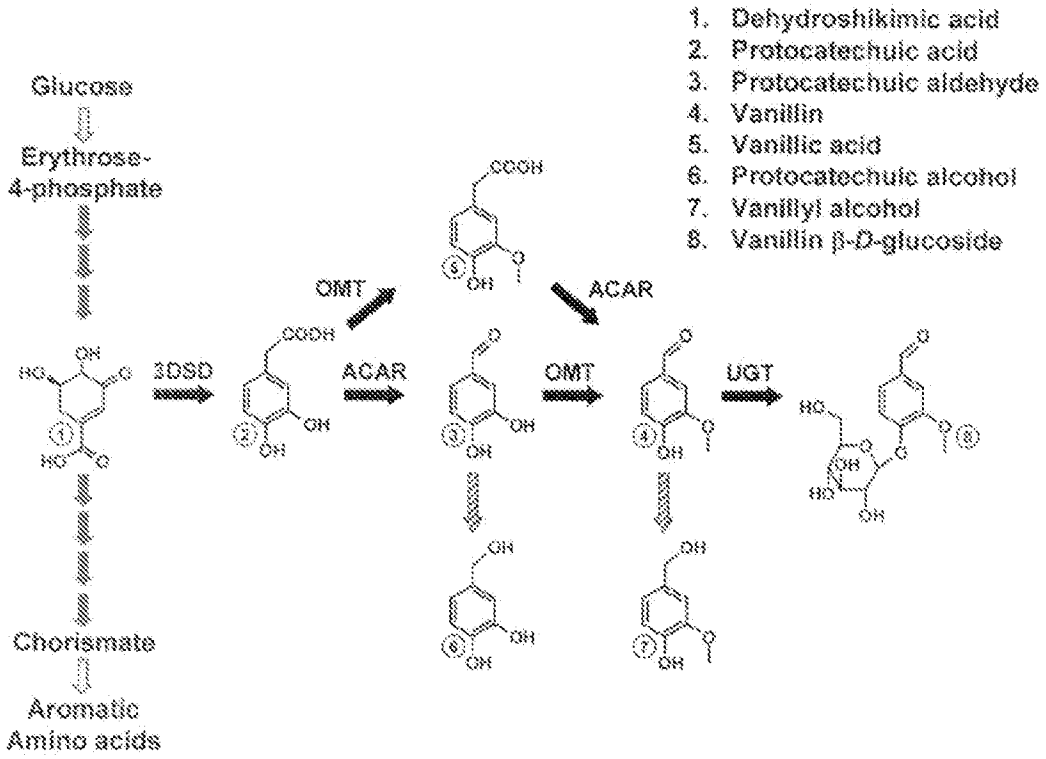

FIG. 1. Biosynthetic scheme for de novo biosynthesis of vanillin in *Schizosaccharomyces pombe* and outline of the different vanillin catabolites and metabolic side products observed in different yeast strains and constructs. Gray arrows, primary metabolic reactions in yeast; black arrows, enzyme reactions introduced by metabolic engineering; diagonally striped arrows, undesired inherent yeast metabolic reactions.

Compared to bioconversion, de novo biosynthesis of vanillin from a primary metabolite like glucose is much more attractive, since glucose costs less than $0.30/kilogram (42). One route for microbial production of vanillin from glucose was devised by Frost and coworker Li (6, 20), combining de novo biosynthesis of vanillic acid in *E. coli* with enzymatic in vitro conversion of vanillic acid to vanillin. 3-Dehydroshikimic acid is an intermediate in the shikimate pathway for biosynthesis of aromatic amino acids, and the recombinant *E. coli* was engineered to dehydrate this compound to form protocatechuic acid (3,4-dihydroxybenzoic acid) and methylate this to form vanillic acid. The vanillic acid was subsequently converted into vanillin in vitro using carboxylic acid reductase isolated from *Neurospora crassa*. The main products of the in vivo step were protocatechuic acid, vanillic acid, and isovanillic acid in an approximate ratio of 9:4:1, indicating a bottleneck at the methylation reaction and nonspecificity of the OMT (*O*-methyltransferase) enzyme for the *meta*-hydroxyl group of protocatechuic acid. Serious drawbacks of this scheme are the lack of an in vivo step for the enzymatic reduction of vanillic acid, demanding the addition of isolated carboxylic acid reductase and costly cofactors such as ATP, NADPH, and Mg^{2+}, and the generation of isovanillin as a contaminating side product.

In this study, we have genetically engineered single-recombination microorganisms to synthesize vanillin from glucose, according to the metabolic route depicted in Fig. 1. To avoid the synthesis of isovanillin as an undesired side product, a large array of OMTs was screened for the desired high substrate specificity, and an appropriate enzyme was identified. A synthetic version of an aromatic carboxylic acid reductase (ACAR) gene, optimized for yeast codon usage, was introduced to achieve the reduction step. The vanillin pathway was introduced into both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* yeast, and significant levels of vanillin production were obtained in both organisms. Vanillin β-D-glucoside is the form in which vanillin accumulates and is stored in the fresh pod of the vanilla orchid (*Vanilla planifolia*). During the "curing" process of the pod, β-glucosidases are liberated and facilitate a partial conversion of the vanillin β-D-glucoside into vanillin. Upon consumption or application, the conversion of vanillin β-D-glucoside into free vanillin by enzymes in the saliva or in the skin microflora can provide for a slow-release effect that prolongs and augments the sensory event, as is the case for other flavor glycosides investigated, such as menthol glucoside (14, 16). In addition to the increased value of vanillin β-D-glucoside as an aroma or flavor compound, production of the glucoside in yeast may offer several advantages. Vanillin β-D-glucoside is more water soluble than vanillin, but most importantly, compounds such as vanillin in high concentrations are toxic to many living cells (4). It has been shown that glucosides of toxic compounds are less toxic to yeasts (24). We found this to be the case with vanillin and *S. cerevisiae* yeast as well. Thus, to facilitate storage and accumulation of higher vanillin yields, we introduced a step for vanillin glucosylation in *S. pombe*.

TABLE 1. Plasmids used in this study

Plasmid name	Gene content	GenBank accession no.	Plasmid type	Selection marker
pSP-fix-Kan				KanMX
pJH606			Integration (*S. pombe*)	*Leu1*$^+$
pJH609			Integration (*S. pombe*)	HphMX
pJH610			Integration (*S. pombe*)	NatMX
pJH573	ACAR (*Nocardia* sp., synthetic codon optimized)	AY495697	Integration (*S. pombe*)	*Leu1*$^+$
pJH643	3DSD (*Podospora pauriseta*)	CAD60599	Integration (*S. pombe*)	KanMX
pJH620	Hs-OMT (*Homo sapiens*, synthetic codon optimized)	NM_000754	Integration (*S. pombe*)	HphMX
pJH622	Ms-OMT (*Medicago sativa*, synthetic codon optimized)	M63853	Integration (*S. pombe*)	HphMX
pJH623	Co-OMT (*Capsicum chinense*)	AF081214	Integration (*S. pombe*)	HphMX
pJH624	At-OMT (*Arabidopsis thaliana*)	AY062837	Integration (*S. pombe*)	HphMX
pJH625	Nt-OMT-1a (*Nicotiana tabacum*)	X74452	Integration (*S. pombe*)	HphMX
pJH627	Nt-OMT-1b (*Nicotiana tabacum*)	X74453	Integration (*S. pombe*)	HphMX
pJH628	Fa-OMT (*Fragaria* × *ananassa*)	AF220491	Integration (*S. pombe*)	HphMX
pJH632	UGT71C2 (*Arabidopsis thaliana*)	AC005496	Integration (*S. pombe*)	NatMX
pJH633	UGT72B1 (*Arabidopsis thaliana*)	NM_116237	Integration (*S. pombe*)	NatMX
pJH665	UGT72E2 (*Arabidopsis thaliana*)	NM_126067	Integration (*S. pombe*)	NatMX
pJH239			*CEN*-ARS (*S. cerevisiae*)	*URA3*
pJH500	3DSD (*Podospora pauciseta*)	CAD60599	Integration (*S. cerevisiae*)	AurC-R
pJH343	Hs-OMT (*Homo sapiens*, synthetic codon optimized)	NM_000754	Integration (*S. cerevisiae*)	NatMX
pJH674	ACAR (*Nocardia* sp., synthetic codon optimized)	AY495697	Integration (*S. cerevisiae*)	HphMX
pJH587	*acpS* (*Escherichia coli*)	NC_000913	*CEN*-ARS (*S. cerevisiae*)	*URA3*
pJH588	*sfp*^{0} (*Escherichia coli*)	NZ_AAEB02000001	*CEN*-ARS (*S. cerevisiae*)	*URA3*
pJH589	*entD* (*Escherichia coli*)	NZ_ABHP01000004	*CEN*-ARS (*S. cerevisiae*)	*URA3*
pJH590	PPTase-1 (*Escherichia coli*)	NC_000913	*CEN*-ARS (*S. cerevisiae*)	*URA3*
pJH591	*acpS* (*Corynebacterium glutamicum*)	NC_003450	*CEN*-ARS (*S. cerevisiae*)	*URA3*
pJH592	PPTase-1 (*Corynebacterium glutamicum*)	NC_003450	*CEN*-ARS (*S. cerevisiae*)	*URA3*
pJH593	*acpS* (*Mycobacterium bovis*)	NC_008062	*CEN*-ARS (*S. cerevisiae*)	*URA3*
pJH594	*pptT* homologue (*Mycobacterium bovis*)	NC_002945	*CEN*-ARS (*S. cerevisiae*)	*URA3*
pJH595	*sfp* (*Bacillus subtilis*)	EU882341	*CEN*-ARS (*S. cerevisiae*)	*URA3*
pJH596	*acpS* (*Bacillus subtilis*)	NC_000964	*CEN*-ARS (*S. cerevisiae*)	*URA3*
pJH701	PPTase-1 (*Nocardia farcinica*, synthetic codon optimized)	NC_006361		

MATERIALS AND METHODS

Isolation and subcloning of genes, and construction of expression cassettes. The 1,104-bp gene sequence of the *Podospora pauciseta* 3-dehydroshikimate dehydratase (3DSD) gene has no introns and was PCR amplified from genomic *P. pauciseta* DNA with flanking XbaI and BamHI restriction sites. The isolated PCR product was subcloned into the pCR-Blunt II-TOPO vector (Invitrogen Corp.), and the sequence-verified gene was inserted in pJH606, a proprietary *S. pombe* expression vector containing the *S. pombe leu1*$^+$ selection marker and the *adh1*$^+$ promoter. The resulting plasmid was named pJH643. The *Nocardia* sp. ACAR gene was synthesized with *S. pombe* codon optimization (to match as closely as possible the average codon usage as defined by all *S. pombe* sequences present in the NCBI-GenBank database) and flanking XbaI and BamHI sites (GENEART GmbH, Germany) and was inserted in the proprietary *S. pombe* expression vector pSP-fix-Kan. This vector contains the KanMX selection marker, conferring resistance to the drug G418, and the *S. pombe adh1*$^+$ promoter for gene expression. The resulting plasmid was named pJH573. The Ms-OMT and Hs-OMT genes were synthesized with *S. pombe* codon optimization (to match as closely as possible the average codon usage as defined by all *S. pombe* sequences present in the NCBI-GenBank database) and flanking XbaI and BamHI sites (GENEART GmbH, Germany). All other OMT-encoding genes were amplified by PCR from cDNA libraries (Stratagene Inc.) or cDNA clones (Co-OMT, courtesy of Mary O'Connell, and Fa-OMT, courtesy of Stefan Lunkenbein) using primers containing flanking XbaI and BamHI sites. After being cloned into pCR-Blunt II-TOPO and sequence verification, the genes were transferred with XbaI and BamHI restriction sites into the proprietary *S. pombe* expression vector pJH609. This vector contains the HphMX selection marker, conferring resistance to hygromycin B, and the *S. pombe adh1*$^+$ promoter for gene expression. The expression plasmids constructed were named as follows: pJH620 (Hs-OMT), pJH622 (Ms-OMT), pJH623 (Co-OMT), pJH624 (At-OMT), pJH625 (Nt-OMT-1a), pJH627 (Nt-OMT-1b), and pJH628 (Fa-OMT). UGT71C2, UGT72B1, and UGT72E2 were all PCR amplified from proprietary *Arabidopsis thaliana* clones (C. Kristensen, E. B. Hansen, T. B. Andersen, G. Kock, F. T. Okkels, B. L. Møller, and I. Hansen, unpublished data) with appropriate flanking restriction sites for insertion in the proprietary *S. pombe* expression vector pJH610 (identical to pJH606 except the *leu1*$^+$ marker is exchanged with a NatMX [nourseothricin resistance] marker). The resulting plasmids were pJH632 (UGT71C2), pJH633 (UGT72B1), and pJH665 (UGT71E2). For expression in *S. cerevisiae*, the 3DSD gene was inserted with XbaI-BamHI in a proprietary derivate of plasmid pYC070 (12), containing the strong constitutive *S. cerevisiae TPI1* promoter and terminator and the AurC-R (aureobasidin A resistance) selection marker. This resulted in plasmid pJH500. The Hs-OMT gene was likewise inserted with XbaI-BamHI into a similar expression vector derived from pYC650 (12) (containing the NatMX selection marker), resulting in plasmid pJH343. The ACAR gene was inserted with XbaI-BamHI into a similar derivative of plasmid pYC380 (12) (containing the HphMX selection marker), resulting in plasmid pJH674. Finally, most PPTase genes were obtained by PCR amplification of genomic DNA from *E. coli*, *Bacillus subtilis*, *Mycobacterium bovis*, and *Corynebacterium glutamicum*, while the *Nocardia farcinica* gene was obtained as a synthetic gene construct optimized for *S. cerevisiae* codon usage (GENEART GmbH, Germany). In all cases, the genes contained flanking XbaI-BamHI or XbaI-BglII (*E. coli acpS*) and were inserted in the XbaI-BamHI sites of the proprietary yeast shuttle (*CEN*-ARS replication) expression vector pJH239 containing the *TPI1* promoter and terminator and the *URA3* selection marker. This resulted in plasmids pJH587 to pJH596 and pJH701. All PCRs were performed using a Peltier thermal cycler DNA engine DYAD PCR machine, with an initial preheating at 94°C for 2 min and a final 7-min elongation step at the selected elongation temperature. *Pwo* polymerase (Roche Biochemicals) was used for all reactions. All plasmids used or constructed are listed in Table 1.

Yeast transformation and selection of transformants. The 3DSD gene expression cassette was transformed into *S. pombe* strain SP687 as a linearized plasmid, pJH643, with integration directed to the *leu1*$^+$ locus. A leucine prototrophic transformant was isolated and denoted strain FSC264, and after confirmation of its ability to produce protocatechuic acid, it was kept as strain VAN264. The ACAR gene expression cassette was transformed into strain VAN264 as linearized plasmid pJH573, with integration directed to the *adh1*$^+$ promoter region. Eight G418-resistant transformants were selected, and the one with the highest total production of protocatechuic acid and aldehyde was kept as strain VAN344. All plasmids containing expression cassettes for OMTs were transformed into strain VAN264 after linearization to direct integration to the *adh1*$^+$ promoter region. Two hygromycin B-resistant transformants of each type were tested for

TABLE 2. Yeast strains used in this study

Yeast strain	Relevant genotype[a]	Reference
Saccharomyces cerevisiae X2180-1A	Wt	Public domain
Saccharomyces cerevisiae VAN100	*his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *adh6::LEU2 bgl1*::KanMX4	This study
Saccharomyces cerevisiae VAN268	*his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *adh6::LEU2 bgl1*::KanMX4 P_{TPI1}::3DSD [AurC]	This study
Saccharomyces cerevisiae VAN277	*his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *adh6::LEU2 bgl1*::KanMX4 P_{TPI1}::3DSD [AurC]::Hs-OMT [NatMX]	This study
Saccharomyces cerevisiae VAN286	*his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *adh6::LEU2 bgl1*::KanMX4 P_{TPI1}::3DSD [AurC]::Hs-OMT [NatMX]::ACAR [HphMX]	This study
Saccharomyces cerevisiae var. *diastaticus* CBS1789	Wt	Centraalbureau voor Schimmelcultures
Saccharomyces bayanus CBS380	Wt	Centraalbureau voor Schimmelcultures
Saccharomyces uvarum CBS395	Wt	Centraalbureau voor Schimmelcultures
Saccharomyces carlsbergensis CBS1513	Wt	Centraalbureau voor Schimmelcultures
Saccharomyces pastorianus CBS1538	Wt	Centraalbureau voor Schimmelcultures
Saccharomyces paradoxus CBS2908	Wt	Centraalbureau voor Schimmelcultures
Saccharomyces globosus CBS424	Wt	Centraalbureau voor Schimmelcultures
Saccharomyces servazzii CBS4311	Wt	Centraalbureau voor Schimmelcultures
Saccharomyces castellii CBS4309	Wt	Centraalbureau voor Schimmelcultures
Saccharomyces kluyveri Y057	Wt	J. Piskur, University of Lund
Zygosaccharomyces fermentati UCBS4506	Wt	Carlsberg Research Center
Zygosaccharomyces bisporus CBS708	Wt	Centraalbureau voor Schimmelcultures
Debaromyces occidentalis CBS819	Wt	Centraalbureau voor Schimmelcultures
Tundaspora delbrueckii Y063	Wt	T. H. Andersen
Kluyveromyces lactis TM4	MATa *trp*$^-$ K$^+$R$^+$	J. Piskur, University of Lund
Pichia pastoris KM71H	*arg4 aox1*::*ARG4*	Invitrogen Inc.
Schizosaccharomyces pombe SP887	*h90 ura4*$^-$ *ade6-210 leu1-32*	Public domain
Schizosaccharomyces pombe VAN244	*h90 ura4*$^-$ *ade6-210 leu1-32*::3DSD [Leu$^+$] *Padh1*::ACAR [G418R]	This study
Schizosaccharomyces pombe VAN264	*h90 ura4*$^-$ *ade6-210 leu1-32*::3DSD [Leu$^+$]	This study
Schizosaccharomyces pombe VAN294	*h90 ura4*$^-$ *ade6-210 leu1-32*::3DSD [Leu$^+$] *Padh1*::ACAR [G418R]::Hs-OMT [HphMX]	This study
Schizosaccharomyces pombe VAN298	*h90 ura4*$^-$ *ade6-210 leu1-32*::3DSD [Leu$^+$] *Padh1*::ACAR [G418R]::At-OMT [HphMX]	This study
Schizosaccharomyces pombe VAN302	*h90 ura4*$^-$ *ade6-210 leu1-32*::3DSD [Leu$^+$] *Padh1*::ACAR [G418R]::Fa-OMT [HphMX]	This study
Schizosaccharomyces pombe VAN512	*h90 ura4*$^-$ *ade6-210 leu1-32*::3DSD [Leu$^+$] *Padh1*::ACAR [G418R]::Hs-OMT [HphMX]::UGT71B2 [NatMX]	This study
Schizosaccharomyces pombe VAN513	*h90 ura4*$^-$ *ade6-210 leu1-32*::3DSD [Leu$^+$] *Padh1*::ACAR [G418R]::Hs-OMT [HphMX]::UGT72B1 [NatMX]	This study
Schizosaccharomyces pombe VAN515	*h90 ura4*$^-$ *ade6-210 leu1-32*::3DSD [Leu$^+$] *Padh1*::ACAR [G418R]::Hs-OMT [HphMX]::UGT72E2 [NatMX]	This study

[a] Wt, wild type.

production of vanillin pathway metabolites, and the one with the highest vanillin production was kept as strain VAN294 (Hs-OMT), VAN298 (At-OMT), or VAN302 (Fa-OMT). The UDP-glycosyltransferase (UGT)-containing plasmids pJH682 (UGT71C2), pJH683 (UGT72B1), and pJH868 (UGT71B2) were all linearized in order to direct integration to the *ade1*$^+$ promoter region, and strain VAN294 was transformed with the plasmid preparations. One stable nourseothricin-resistant transformant of each type was kept as strains VAN512 (UGT71C2), VAN513 (UGT72B1), and VAN515 (UGT72E2). Plasmid pJH680 was linearized with BsaI-HF in order to direct integration to the *TPI1 promoter* region, and *S. cerevisiae* strain VAN100 (*adh6 bgl2*) was transformed with the plasmid preparation. One PCR-confirmed, aureobasidin A-resistant transformant was kept as strain VAN268. This strain was transformed with BsaI-HF-linearized plasmid pJH543. One PCR-reconfirmed, nourseothricin-resistant transformant was kept as strain VAN277. Strain VAN277 was transformed with BsaI-HF-linearized plasmid pJH674, and one PCR-reconfirmed, hygromycin B-resistant transformant was kept as strain VAN286. All yeast strains used or created in this study are listed in Table 2. *S. pombe* and *S. cerevisiae* were transformed with plasmid DNA using the respective lithium acetate methods for these two organisms (7, 29), and the proper insertion of all expression cassettes at the desired genomic location was confirmed by analytical PCR on genomic material from the various yeast strains.

In vivo test for vanillin and vanillin β-D-glycoside reduction and for production of vanillin biosynthesis pathway metabolites. Yeast strains were in all cases grown at 25°C with 170-rpm shaking in appropriate growth media (synthetic complete [SC] or yeast extract-peptone-dextrose [YPD] for *S. cerevisiae* strains, yeast extract with supplements [YES] for *S. pombe* strains), after inoculation from precultures grown under the same conditions. No precautions were taken to avoid the presence of aromatic amino acids in these growth media, which potentially could limit dehydroshikimic acid biosynthesis. Growth media were in all cases obtained from Q-BioGene, Montreal, Canada. To analyze the rate of turnover of vanillin and vanillin β-D-glucoside in the yeast cultures, these compounds were supplied in final concentrations of 1 mM from 1 M stock solutions in ethanol (the yeast strains analyzed are listed in Table 3). For metabolite analysis, the ferment of growth culture samples was separated from the yeast cells by centrifugation. Ferment (500 μl) was then combined with 500 μl of 100% methanol and recentrifuged (16,100 × *g*, 10 min) to precipitate macromolecules. Aliquots (25 μl) were analyzed by high-performance liquid chromatography (HPLC) as described below.

Analysis of the growth-inhibitory effect of vanillin and vanillin β-D-glucoside on yeast. A preculture of *S. cerevisiae* strain VAN100 in SC medium (optical density at 600 nm [OD_{600}] of approximately 2.5) was diluted into two equal batches (300 ml) of the same medium (OD_{600} of 0.4, 250-ml Erlenmeyer flasks). Vanillin was added to the flasks in final concentrations of 0.5, 1.0, and 5 g/liter. Vanillin β-D-glucoside was added in a final concentration of 25 g/liter to a fourth culture, while a fifth culture to which neither vanillin nor vanillin β-D-glucoside was added was used as a control. The cultures were grown at 25°C with 150-rpm shaking, and the OD_{600} was measured after 5.5, 9.5, and 23 h.

Extraction and purification of vanillin from large-scale batch cultures. Vanillin was extracted from supernatants of large-scale yeast cultures using CH_2Cl_2 in three serial extractions (333 ml per 1 liter of supernatant). The extract was concentrated in a rotary evaporator, the residue was resuspended in toluene, and the suspension was applied to a silica gel column, which was eluted with 30% ethyl acetate in pentane. The fractions containing vanillin, as monitored by thin-layer chromatography and their UV fluorescence were combined and concentrated by drying in a rotary evaporator.

HPLC analysis. Intermediates in vanillin biosynthesis and vanillin catabolites were analyzed using an Agilent 1100 series HPLC system using a Zorbax SB-C18 column (4.6 by 150 mm, 3.5-μm particle size). The elution buffer was a gradient of acetonitrile (MeCN) and H_2O (adjusted to pH 2.3 with H_2SO_4) composed as follows: 0% to 40% MeCN for 3 min, 40% MeCN for 1 min, 40% to 80% MeCN for 2 min, and 80% to 90% MeCN for 1 min. The temperature of the solvent was thermostated at 30°C, and a diode array detector was used to detect eluted compounds by their UV fluorescence at 210 nm and 280 nm. Vanillin, protocatechuic acid, protocatechuic aldehyde, vanillic acid, and vanillyl alcohol standards were obtained from Merck Chemical Co. Vanillin β-D-glucoside was obtained from Apin Chemicals Ltd., United Kingdom.

NMR analysis. Nuclear magnetic resonance (NMR) spectra were recorded in deuterated chloroform on a Bruker Avance 400 instrument using tetramethyl silane as an internal standard. The ^1H spectrum exhibited the following signals: 9.82 ppm (CHO), multiplets at 7.43 (2H) and 7.04 (1H) (aromatic protons), and 3.95 ppm (CH_3O). The ^{13}C spectrum showed signals at 191.0 (CHO), 151.8, 147.8, 129.8, 127.5, 114.5, and 108.9 (aromatic carbons) and 56.1 ppm (CH_3O). The ^1H and ^{13}C spectra were identical to those of authentic vanillin and clearly different from those of isovanillin, which among other signals had ^{13}C signals at 124.6 and 116.2 ppm and a multiplet at 6.98 (1H) in the ^1H spectrum.

RESULTS

***Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are both appropriate hosts for vanillin biosynthesis.** The production organism was chosen based on the evaluation of several parameters: (i) GRAS ("generally regarded as safe") recognition, (ii) proven suitability in at least one established production system, (iii) reasonably well developed genetic tools available, and (iv) inherent vanillin metabolism that is as low as possible. From a genetic point of view, the most obvious candidates were strains of baker's yeast (*Saccharomyces cerevisiae*) and *Escherichia coli*. These are GRAS organisms and constitute well-known production systems, their genome sequences are available, and genetic manipulation is relatively straightforward. From a consumer acceptance point of view, *S. cerevisiae* would appear to be the best choice. However, a growing culture of *S. cerevisiae* (laboratory strain X2180-1A) quantitatively reduced externally added vanillin (1 mM) to vanillyl alcohol within 48 h (data not shown). This prompted us to test a range of different yeast species of the genus *Saccharomyces*, along with strains of *Zygosaccharomyces fermentati*, *Zygosaccharomyces bisporus*, *Debaryomyces occidentalis*, *Torulaspora delbrueckii*, *Kluyveromyces lactis*, *Pichia pastoris*, and *Schizosaccharomyces pombe* (Table 2). *Schizosaccharomyces pombe* was by far the most satisfactory, since after 48 h it had reduced less than 50% of the vanillin provided and oxidized none (data not shown), whereas all other strains tested converted all vanillin to either vanillyl alcohol or vanillic acid within the same period of time. In a similar manner, we tested hydrolysis of vanillin β-D-glucoside by *S. pombe* and *S. cerevisiae*. While *S. pombe* left vanillin β-D-glucoside intact even after prolonged incubation, *S. cerevisiae* hydrolyzed all vanillin β-D-glucoside within 24 h (data not shown). This in turn prompted us to test *S. cerevisiae* mutants of known β-glucosidase genes (*ALF2*, *BGL1*, *BGL2*, *DSE2*, *DSE4*, *EXG2*, *KRE6*, *SCW10*, *SCW11*, *SCW4*, *SKN1*, *SPR1*, *SUN4*, and the homologous gene YOL155C; mutants were obtained from the Euroscarf collection). One mutant, the *bgl1* strain, hydrolyzed less than 5% of the vanillin β-D-glucoside present, while all other mutants had the same activity as the wild-type yeast (data not shown). Finally, we tested whether *S. cerevisiae* mutants in any of the 29 known or hypothesized alcohol dehydrogenases, aryl-alcohol dehydrogenases, or the related aldose reductases (*AAD3*, *AAD4*, *AAD6*, *AAD10*, *AAD14*, *ADH1*, *ADH2*, *ADH3*, *ADH4*, *ADH5*, *ADH6*, *ADH7*, *ARA1*, *ARA2*, *BDH1*, *BDH2*, *GCY1*, *GRE3*, *SFA1*, *XYL2*, *YPR1*, *ZTA1*, YCR102c, YDL124w, YJR096w, YLR460c, YNL134c, YPL088w, and YPR127w; mutants were obtained from the Euroscarf collection) had a reduced ability to convert vanillin into vanillyl alcohol. The screen identified *ADH6* as the most important gene encoding a vanillin reductase (data not shown). Consequently, we bred an *adh6* mutant of *S. cerevisiae bgl1* strain YI5210 (Euroscarf), strain VAN100 (Table 2). This strain grew normally under all circumstances tested, hydrolyzed vanillin β-D-glucoside to only a very limited extent, and showed a 50%-decreased ability to reduce vanillin to vanillyl alcohol. Thus, we decided to test vanillin biosynthesis in a wild-type *S. pombe* yeast and in the *bgl1 adh6* mutant of *S. cerevisiae*.

A de novo vanillin biosynthesis pathway can be constituted in *S. pombe* yeast by the expression of three heterologous genes. 3DSD catalyzes the conversion of 3-dehydroshikimic acid to protocatechuic acid. This enzyme activity is known from filamentous fungi (40), so we isolated the gene encoding this enzyme from the dung mold *Podospora pauciseta*. The gene was PCR isolated from genomic DNA and transformed into *S. pombe* strain SP887 on the linearized pJH643 *S. pombe* expression plasmid. One transformant, denoted strain VAN264 (Table 2), was isolated and tested for its ability to produce protocatechuic acid by growing a batch culture (5 ml) for 48 h, after which the supernatant was analyzed by HPLC. A new compound eluting at 5.4 min was identified as protocatechuic acid based on its coelution with authentic protocatechuic acid and an identical absorption spectrum. The production of protocatechuic acid reached more than 360 mg/liter (Table 3).

ACARs (EC 1.2.1.30) catalyze the ATP-driven reduction of protocatechuic acid to protocatechuic aldehyde. Bacteria of the *Nocardia* genus as well as filamentous and ligninolytic fungi are known to possess this enzyme activity (9, 11, 21), and a method to reduce vanillic acid to vanillin using purified *Nocardia* ACAR enzyme was devised by Rosazza and Li (39). The corresponding 3.5-kb ACAR gene has been isolated, and a recombinant *E. coli* strain expressing the enzyme bioconverts vanillic acid to vanillin (13). The codon GC content in the *Nocardia* genus is around 70%, while it is a more 40% in *S. pombe*. To optimize expression, a synthetic version of the gene was built based on *S. pombe* codon usage and transformed into *S. pombe* strain VAN264 on the linearized expression plasmid pJH573. Eight transformants were grown in batch cultures (5

TABLE 3. Production of vanillin and intermediates in in vivo experiments[a]

Strain	Production (mg/liter)				
	Vanillin	Vanillyl alcohol	Vanillic acid	Protocatechuic acid	Protocatechuic aldehyde
VAN264	ND	ND	ND	364	ND
VAN244	ND	ND	ND	140 (30)*	160 (50)*
VAN298	20.3 (0.1)**	11.5 (0.4)**	ND	87 (2)**	98 (3)**
VAN302	37 (6)**	28 (10)**	ND	50 (3)**	40 (7)**
VAN294	65 (6)**	24 (14)**	16 (3)**	15 (2)**	5 (3)**
VAN286 [PPTcg-1]	46 (2)**	111 (10)**	20.2 (1.2)**	52 (5)**	12.9 (0.5)**

[a] HPLC analysis of supernatants of 5-ml cultures grown for 48 h. ND, not detected; *, standard deviation of eight independent clones tested; **, statistical crude range of values from two independent clones tested. *S. pombe* strains (VAN264, VAN244, VAN298, VAN302, and VAN294) were grown in rich YES medium, while *S. cerevisiae* strains (VAN286 [PPTcg-1]) were grown in SC medium.

ml) for 48 h, the cells were removed by centrifugation, and the supernatant was analyzed by HPLC. In addition to protocatechuic acid, a new constituent was found to elute at 5.8 min and was identified as protocatechuic aldehyde, based on coelution with an authentic standard and spectral analysis. The transformant with the highest total production of protocatechuic acid plus protocatechuic aldehyde afforded 300 mg/liter and was kept as strain VAN244 (Table 2). This strain converted 53% of the formed protocatechuic acid into protocatechuic aldehyde (Table 3).

Two OMTs, from alfalfa (*Medicago sativa*) and strawberry (*Fragaria* × *ananassa*) (Ms-OMT and Fa-OMT) (5, 47), were reported to catalyze 3′-OH position-specific methylation of protocatechuic aldehyde. Based on the sequence information for these genes, similar OMT genes from *Capsicum chinense* (Cc-OMT), *Arabidopsis thaliana* (At-OMT), and *Nicotiana tabacum* (Nt-OMT-a1 and -b1) were isolated. All of the genes encoding these proteins are approximately 1,100 bp. A different class of methyltransferase-encoding genes of approximately 700 bp, widespread in animals, is annotated as catechol methyltransferase. For comparative purposes, we expanded the screen with a human (*Homo sapiens*) catechol methyltransferase (Hs-OMT) (18). The OMT-encoding genes were PCR amplified from cDNA or synthesized with *S. pombe* codon optimization (Ms-OMT and Hs-OMT) and transformed into *S. pombe* strain VAN244 as linearized plasmids pJH620 (Hs-OMT), pJH622 (Ms-OMT), pJH623 (Cc-OMT), pJH624 (At-OMT), pJH625 (Nt-OMT-a1), pJH627 (Nt-OMT-b1), and pJH628 (Fa-OMT) (Table 1). Two of each type of transformant were grown in batch cultures (5 ml) for 48 h, and the supernatants were analyzed by HPLC. Only expression of Hs-OMT, At-OMT, and Fa-OMT resulted in in vivo methylation, measured as the accumulation of vanillic acid (elution time, 5.9 min) and/or vanillin (elution time, 6.6 min) and confirmed by comparison of the elution profile and absorbance with authentic standards. One strain expressing each of these OMTs was kept: VAN294 (Hs-OMT), VAN298 (At-OMT), and VAN302 (Fa-OMT). The three OMTs afforded quite different product profiles (Fig. 2 and Table 3). VAN298, carrying At-OMT, produced the smallest amount of vanillin, despite the fact that the level of the precursors protocatechuic acid and protocatechuic aldehyde were the highest in this strain. VAN302, carrying Fa-OMT, produced nearly twice as much vanillin. VAN294, expressing the human catechol methyltransferase (Hs-OMT), was by far the most efficient enzyme and more than tripled the amount of vanillin made by VAN298. VAN294 also produced vanillyl alcohol (elution time, 5.5 min) and vanillic acid (elution time, 6.2 min). Because of the singularly high vanillin formation in the VAN294 strain, harboring expression cassettes for 3DSD, ACAR, and Hs-OMT, this strain was chosen for vanillin production.

Small-scale vanillin production was performed using strain VAN294. Cultures (four at 3 liters each) were started from precultures (OD_{600} of 0.04) in rich medium and allowed to

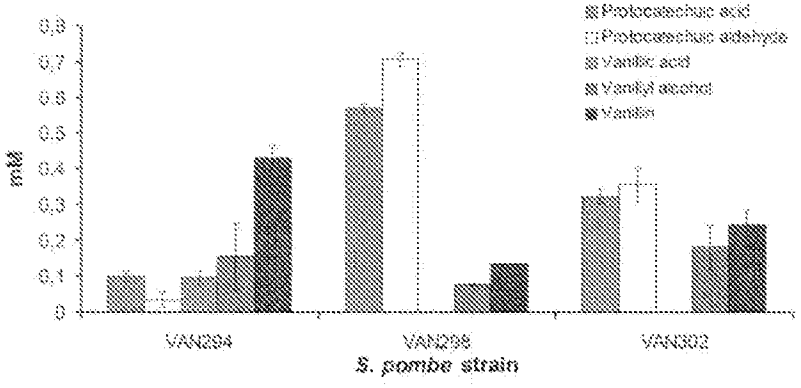

FIG. 2. Accumulation of vanillin, vanillin catabolites, and intermediates in vanillin biosynthesis in three vanillin-producing *S. pombe* strains (values correspond to those in Table 3).

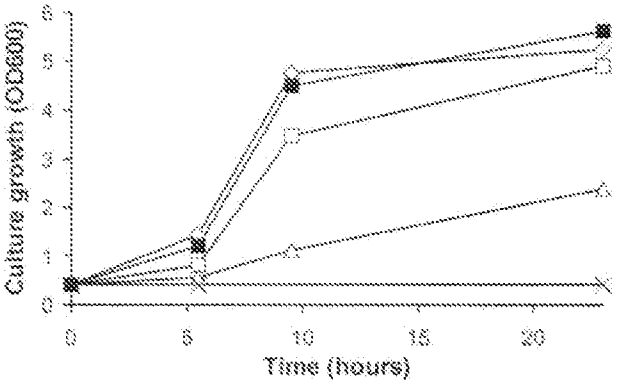

FIG. 3. Toxicity for growth of *Saccharomyces cerevisiae* of vanillin and vanillin β-D-glucoside. *S. cerevisiae* strain VAN100 was grown for 23 h at various concentrations of vanillin (open squares, 0.3 g/liter; open triangles, 1 g/liter; crosses, 5 g/liter), of vanillin β-D-glucoside at 25 g/liter (closed squares), or without either compound (open diamonds).

grow for 48 h (vanillin production ceased after 45 h). Vanillin content in the four culture flasks varied between 21 mg/liter and 31 mg/liter, corresponding to a total production of approximately 300 mg of vanillin in the 12 liters of culture. Invariably, a reduced yield of vanillin was observed when the culture volume was increased. Currently, the reduced yield cannot be related to specific growth parameters. Extraction of the cleared culture supernatant with CH_2Cl_2 (as described in Materials and Methods) afforded approximately 200 mg of vanillin as white powder. The isolated vanillin showed an HPLC elution time and UV spectrum indistinguishable from those of a vanillin standard and an NMR spectrum identical to that of authentic vanillin (NMR signals reported in Materials and Methods). The NMR analysis documented that no isovanillin (3-hydroxy-4-methoxybenzaldehyde) was present.

Additional expression of a plant family 1 UGT results in de novo biosynthesis of vanillin β-D-glucoside. The successful design of a de novo pathway for vanillin biosynthesis in *S. pombe* prompted us to investigate the possibility of converting the vanillin formed into vanillin β-D-glucoside. This set of experiments was further accentuated by the observation that the glucosylated form of vanillin was less toxic to yeast than vanillin. The growth-inhibitory effects of the two compounds were tested using the *S. cerevisiae* strain VAN100 (Fig. 3). Whereas vanillin was toxic at a concentration of less than 0.5 g/liter, as monitored by growth inhibition, vanillin β-D-glucoside was nontoxic even at 25 g/liter. The reduced toxicity of vanillin β-D-glucoside in comparison to vanillin was not caused by an inability of the yeast cells to take up vanillin β-D-glucoside, as demonstrated by analysis of the intracellular content of vanillin β-D-glucoside after 48 h of growth in the presence of 10 or 25 g/liter. In both experiments, the intracellular concentration of vanillin β-D-glucoside was approximately twice that found in the growth supernatant (data not shown). Accordingly, we conclude that vanillin β-D-glucoside is truly nontoxic to *S. cerevisiae* even at high concentrations. Plant family 1 glycosyltransferases are involved in the glycosylation of bioactive plant natural products. They belong to a group of glycosyltransferases often referred to as the UGTs, because they transfer sugar moieties (most often glucose) from UDP-bound sugars to low-molecular-mass aglycons (30, 33). To provide a platform for glycosylation of bioactive aglycons, we cloned and heterologously expressed 98 UGT enzymes from the plant *Arabidopsis thaliana* along with a few from other plant sources (Kristensen et al., unpublished). Following expression in the yeast *Pichia pastoris*, we tested crude enzyme preparations for their ability to catalyze in vitro glucosylation of vanillin. Seven UGTs were identified as possessing particularly high in vitro catalytic activity toward vanillin, namely, UGT71C2, UGT72B1, UGT72E2, UGT84A2, and UGT89B1 from *A. thaliana*, UGT85B1 from *Sorghum bicolor* (17), and arbutin synthase from *Rauwolfia serpentina* (2). Of these seven enzymes, the first three exhibited the highest affinity for vanillin. The genes encoding these UGTs were inserted into the *S. pombe* vanillin producer to examine their in vivo functions. The UGT-encoding genes were combined with the *TPI1* promoter in the expression plasmids pJH632 (UGT71C2), pJH633 (UGT72B1), and pJH665 (UGT72E2) (Table 1), and each was integrated into the *ade1*$^+$ locus of strain VAN294. The three resulting strains (VAN512, VAN513, and VAN515) were tested by growing them for 48 h in 100 ml of YES medium in Erlenmeyer flasks. Strain VAN515, harboring UGT72E2, was by far the most efficient in vivo vanillin glucosyltransferase. Figure 4 shows the results of the ensuing HPLC analyses of the ferment from growth of the VAN515 strain and the control strain VAN294. Production of vanillin β-D-glucoside was verified by

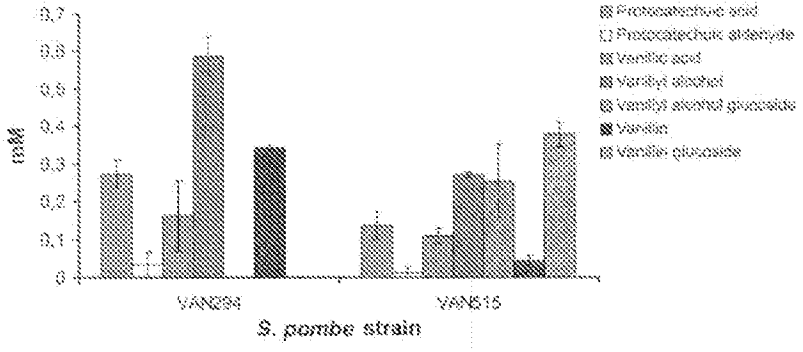

FIG. 4. Accumulation of vanillin, vanillin catabolites, intermediates, and glucosides in vanillin-producing *S. pombe* strain VAN294 alone or with coexpression of UGT72E2 (strain VAN515). The numbers are averages of three experiments.

the elution time of 5.3 min and NMR and UV/visible light spectral identity with a vanillin β-D-glucoside standard. With strain VAN515, a total of 56% of the total vanillin potential (i.e., the sum of formed vanillin and its precursors protocatechuic acid, protocatechuic aldehyde, and vanillic acid) was transformed into vanillin glucoside. Interestingly, while about 80% of the vanillin was glucosylated, only half of the vanillyl alcohol was, confirming a much higher affinity of UGT72E2 for vanillin than for vanillyl alcohol.

Construction of a vanillin-producing *S. cerevisiae* yeast requires heterologous activation of the ACAR gene. The vanillin engineering studies reported above were accomplished with *Schizosaccharomyces pombe* as the host. Because *S. cerevisiae* is the more commonly used "workhorse" for metabolic engineering and production, a parallel study was performed in an attempt to construct an *S. cerevisiae* strain that would also produce vanillin. As in the studies with *S. pombe*, the *P. pauciseta* 3DSD, the synthetic *Nocardia* ACAR, and the synthetic human Hs-OMT genes were all inserted in proprietary *S. cerevisiae* expression cassettes, in all cases making use of the strong glycolytic *TPI1* gene promoter (resulting in integration plasmids pJH500, pJH543, and pJH674 [Table 1]). The expression cassettes were sequentially inserted into the endogenous *TPI1* locus of strain VAN100, directing insertion by the linearization of plasmids in the *TPI1* promoter sequence, resulting in *S. cerevisiae* strain VAN286 (Table 2). After growth of this strain in batch cultures (5 ml) with SC medium for 48 h, the clarified medium was found to contain the vanillin precursors protocatechuic acid and vanillic acid. However, none of the corresponding aldehydes, including vanillin, was detected. This indicated that the ACAR enzyme was not expressed or not functional in *S. cerevisiae*. ACARs as well as the related nonribosomal peptide synthetases, fatty acid synthetases, and polyketide synthetases require specific phosphopantetheinylation for functionality (13, 44). Obviously, an endogenous activity mediating phosphopantetheinylation of ACAR proceeded in the *S. pombe* strain, whereas this activity was absent in *S. cerevisiae*. Consequently, we cloned phosphopantetheine transferases from *Bacillus subtilis* (*acpS* and *sfp*), *E. coli* (*acpS*, *acpT*, *entD*, and a homologue, PPTec-1), *Mycobacterium bovis* (*acp3* and a *sppT* homologue), and *Corynebacterium glutamicum* (*acpS* and PPTcg-1), as well as a homologue from *Nocardia farcinica* (PPTnf-1, a synthetic gene optimized for *S. cerevisiae* codon usage), and expressed these in strain VAN286 from low-copy-number-replicating plasmids (*CEN-ARS*) and the yeast *TPI1* promoter. The *M. bovis* genes were included because *Mycobacterium* is a genus closely related to *Nocardia*, the source of the ACAR gene. Expression of three of the genes, the *E. coli entD*, the *C. glutamicum* PPTcg-1, and the *N. farcinica* PPTnf-1 gene in strain VAN286 (thus harboring either plasmid pJH589, pJH592, or pJH701), resulted in a functional ACAR enzyme and the identification of protocatechuic aldehyde as well as vanillin in the clarified fermentation broth. PPTcg-1 was the most efficient PPTase for activation of the ACAR gene and resulted in formation of 45 mg/liter of vanillin after 48 h of growth in SC medium (Table 3). Thus, the three-step biosynthesis pathway for de novo vanillin biosynthesis already established in *S. pombe* is just as efficient in *S. cerevisiae*, but in contrast to the situation in *S. pombe*, a heterologous PPTase enzyme is needed for activation, by phosphopantetheinylation, of the ACAR gene in *S. cerevisiae*.

DISCUSSION

In this study, we demonstrate complete de novo vanillin production outside the *Vanilla planifolia* seed pod or other plants. This represents the first example of one-cell microbial generation of this valuable compound from glucose, at a production level scalable to industrial needs. The capability for vanillin biosynthesis was introduced into two common yeast species, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. The heterologous pathway for vanillin biosynthesis was engineered in both organisms by the expression of three genes, one from a mold, one from a bacterium, and one of human origin, and in the case of *S. cerevisiae*, one additional bacterial gene. We obtained a vanillin production of 65 and 45 mg/liter in *S. pombe* and *S. cerevisiae*, respectively, free of contaminating isomers, without any specific optimization of media and growth conditions. Although vanillin biosynthesis was less efficient in *S. cerevisiae* than in *S. pombe*, our data actually indicate a higher vanillin production potential in *S. cerevisiae*, since the combined production of vanillin and its precursors and metabolites was almost twice as high with *S. cerevisiae* as with *S. pombe* (Table 3). The accumulated levels of the various metabolites indicate that more dehydroshikimic acid is converted to protocatechuic acid in our *S. cerevisiae* experiment but also that about the same proportion of this (70% for *S. cerevisiae*, 75% for *S. pombe*) is reduced by the introduced ACAR enzyme. The reason for the lower production of vanillin in *S. cerevisiae* is a higher ability of this organism to reduce vanillin to its corresponding alcohol. This undesired property of *S. cerevisiae* became obvious at the beginning of the project and was addressed by inactivation of the *ADH6*-encoded alcohol dehydrogenase. In the set of experiments undertaken to identify the importance of different alcohol dehydrogenases in vanillin reduction, a modest effect of inactivation of several other genes (e.g., *ADH7*) was registered, and it is likely that inactivation of additional alcohol dehydrogenases in the *S. cerevisiae* vanillin producer would result in a significant increase in vanillin production.

The observation that nearly identical proportions of the biosynthesized protocatechuic acid were reduced by both yeast strains demonstrates that introduction of the *C. glutamicum* PPTase gene in our *S. cerevisiae* vanillin producer resulted in an activation of the ACAR enzyme to the same level as that seen in *S. pombe*. It is indeed puzzling that bacterial ACAR can be activated by inherent enzymes in one yeast but not in another. Enzymes requiring phosphopantetheinylation for activation are not abundant in these yeast species, but one well-known example present in both is α-aminoadipate reductase. Both species carry a known PPTase activity taking care of this (Lys5p in *S. cerevisiae*, Lys7p in *S. pombe*), and these are obvious candidates for heterologous ACAR activation (though another could be the PPTase activating mitochondrial fatty acid synthase). A plausible explanation for the differences in PPTase activity in the two yeasts is derived from the following observations (10). Whereas *S. pombe* α-aminoadipate synthase can be activated by PPTases present in *E. coli*, this is not the case for α-aminoadipate synthase from *Candida albicans*. The

C. albicans enzyme is much more closely related to the *S. cerevisiae* enzyme than to the *S. pombe* enzyme. Turning the argument around, this may imply that *S. pombe* (via its *lys7*$^+$-encoded PPTase), but not *S. cerevisiae*, has the inherent ability to activate the bacterial ACAR enzyme. Not surprisingly, a PPTase from *Corynebacterium glutamicum*, a high-GC, gram-positive bacterium related to *Nocardia* sp., turned out to be the most efficient in ACAR activation.

As previously outlined, vanillin β-D-glucoside is the storage form of vanillin found in the *Vanilla* pod. It is nontoxic to most organisms, including yeast, and has a higher solubility in water than does vanillin. In addition, the formation of vanillin β-D-glucoside most likely pulls the biosynthesis further in the direction of vanillin production. The *Arabidopsis thaliana* UDP-glucose glycosyltransferase UGT72E2 exhibited high substrate specificity toward vanillin. In concordance with this observation, its expression in the vanillin-producing *S. pombe* strain resulted in almost all vanillin being converted into vanillin β-D-glucoside. The ability to turn vanillin into vanillin β-D-glucoside in vivo is very important, because microbial production of nonglucosylated vanillin beyond the 0.5- to 1-g/liter scale would be hampered by the toxicity of free vanillin. Glucosylation would serve to circumvent the inhibitory effect. Although glucosylation did not give rise to a major increase in vanillin production, the content of nonmethylated intermediates (protocatechuic acid and aldehyde) was reduced by more than 50% (Fig. 4). This indicates that glucosylation does indeed drive production of methylated vanillin equivalents, but that only a certain amount of dehydroshikimic acid is available during the period of time when our introduced vanillin pathway is active. There could be many reasons for this and we are currently studying several possibilities.

"Sustainable" and "renewable" biological production systems are attracting a lot of attention these days, due to the global warming issue and associated interest in developing a chemical industry that is independent of fossil fuel starting materials; thus, "white biotechnology" is having a tremendous comeback. *S. cerevisiae* is a very attractive production organism in white biotechnology, because this yeast species is well characterized, is easy to manipulate and grow, and has gained GRAS status. Metabolic engineering of *S. cerevisiae* has resulted in very high yields of certain primary yeast metabolites, e.g., 153 g/liter of pyruvate (43), but de novo productivities of novel metabolites have usually been quite modest, ranging from 153 mg/liter (the terpenoid amorphadiene [38]) to only just detectable amounts (e.g., the polyketide precursor methylmalonyl-coenzyme A [26]) (reviewed in reference 28). To our knowledge, our study is the first in which aromatic amino acid biosynthesis intermediates are used for production of a novel compound, and in that perspective, we find our initial productivity of 45 mg/liter satisfactory. We are aware, however, that even though the market prices for "natural" vanillin and for vanillin-β-D-glucoside are high, the biological production system presented here needs to be improved significantly to offer a truly sustainable alternative. It was recently shown that simple genetic modifications may increase the metabolic flux through the *S. cerevisiae* aromatic amino acid biosynthesis pathway 4.5-fold and the extracellular concentration of shikimic acid (the direct metabolite of dehydroshikimic acid) more than 200-fold (22). This provides obvious opportunities for significant future increases in vanillin production using yeasts as production organisms.

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EXHIBIT B

IN BRIEF

INDUSTRIAL MICROBIOLOGY

De novo biosynthesis of vanillin in fission yeast (*Schizosaccharomyces pombe*) and baker's yeast (*Saccharomyces cerevisiae*)

Hansen, E. H. *et al. Appl. Environ. Microbiol.* 13 Mar 2009 (doi:10.1128/AEM.02681-08)

Most vanillin, the compound in vanilla that gives it its flavour, is produced from petrochemicals or wood pulp lignins. Hansen and colleagues have now produced strains of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* that can produce vanillin. They first searched for strains that did not convert vanillin to vanillyl alcohol. They then added genes from the dung mould *Podospora pauciseta*, a bacterium of the *Nocardia* genus, and humans, which allowed the yeast strains to produce vanillin (an additional gene from *Corynebacterium glutamicum* was added to *S. cerevisiae* to activate the *Nocardia* enzyme). At 45–65 mg per litre, vanillin production was at a sufficient level to scale up for large-scale industrial production. These *de novo* pathways for vanillin synthesis in yeast represent the first examples of one-cell microbial generation of these valuable compounds from glucose.

PARASITOLOGY

Influence of ecto-nucleoside triphosphate diphosphohydrolase activity on *Trypanosoma cruzi* infectivity and virulence

Santos, R. F. *et al. PLoS Negl. Trop. Dis.* 3, e567 (2009)

ATP is an important signalling molecule in the host response to pathogens. Many pathogens, including the eukaryotic parasite *Trypanosoma cruzi*, produce an ecto-nucleoside triphosphate diphosphohydrolase (ecto-NTPDase) that decreases extracellular ATP levels in the human host, thereby decreasing the immune response. Santos and colleagues now show that this enzyme plays an important part in *T. cruzi* infections. Three inhibitors of ecto-NTPDase each decreased *T. cruzi* infectivity. However, recombinant *T. cruzi* NTPDase 1 could be inhibited by only one of the three inhibitors, indicating that *T. cruzi* produces additional ecto-NTPDase enzymes. Ecto-NTPDase could therefore be an important new target for drugs against *T. cruzi*.

BACTERIAL PHYSIOLOGY

RNase E autoregulates its synthesis in *Escherichia coli* by binding directly to a stem-loop in the *rne* 5′ untranslated region

Schuck, A., Silva, A., Belasco, J. G. *et al. Mol. Microbiol.* 9 Mar 2009 (doi: 10.1111/j.1365-2958.2009.06662.x)

RNase E plays an important part in the breakdown of mRNA and the maturation of tRNA and rRNA in bacteria, as it cuts RNA into single-stranded regions that are AU-rich. Because alterations in the concentration of the enzyme have detrimental effects on the cell, enzyme production is tightly regulated, in part through processing of the RNase E mRNA by RNase E itself. Schuck and colleagues show that the enzyme binds to the conserved hp2 stem loop in RNase E mRNA, yet cleaves that stem loop poorly. The authors speculate that this binding facilitates RNase E cleavage of the mRNA at other sites. Their findings help to clarify the mechanism by which hp2 mediates feedback regulation of RNase E levels.

EXHIBIT C

Bird for the Band • Sex Roles Lose Appeal • Battling Hepatitis C

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SPECIAL ASTRONOMY ISSUE

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Yeast bred to bear artificial vanilla

Scientists co-opt fungi to produce flavor more efficiently

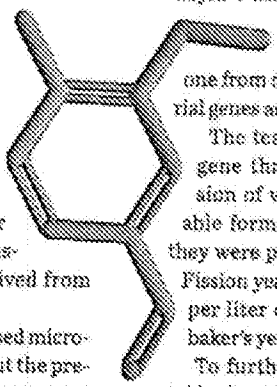
By Rachel Ehrenberg

A jug of wine, a loaf of bread and now, vanilla.

Yeast has long been pressed into service for making food and drink, and now scientists have recruited the fungus for a loftier flavor: vanillin, vanilla's dominant compound. Scientists report in the May *Applied and Environmental Microbiology* that they have engineered two strains of yeast to produce vanillin from glucose, a greener and cheaper route than previous methods.

"This is absolutely beautiful work," says John Rosazza, a medicinal and natural products chemist at the University of Iowa in Iowa City. There is a huge market for vanillin, Rosazza says.

Vanillin is the dominant compound of the hundreds that are found in vanilla — an extract from the seed-bearing pods, called beans, of orchids in the genus *Vanilla*. But real vanilla beans are precious, rare and costly. Today, less than 1 percent of the vanillin sold each year is derived from the orchids. The majority is synthesized in chemistry labs, and typically made from lignin, a constituent of wood left over from the paper-making industry, or guaiacol, which is derived from wood creosote.



Two species of yeast have been engineered to make vanillin (right), the dominant flavor compound in vanilla.

Scientists previously have used microorganisms to make vanillin, but the precursors are expensive and the process involves environmentally unfriendly chemicals, says Jørgen Hansen of Evolva Biotech's Copenhagen office. Also, vanillin itself is toxic to many microbes.

Now Hansen, Birger Lindberg Møller of the University of Copenhagen and colleagues have created a chemistry lab within two different species of yeast growing in flasks: *Schizosaccharomyces pombe*, also known as fission yeast, and *Saccharomyces cerevisiae*, baker's or brewer's yeast. Instead of using the typical, expensive starting material, the team turned to glucose, a cheap and available sugar. To make the yeast convert the glucose to vanillin, the team added genes that encode for specific enzymes that spur the biochemical reactions. These genes included versions of one from a dung mold, two bacterial genes and a human gene.

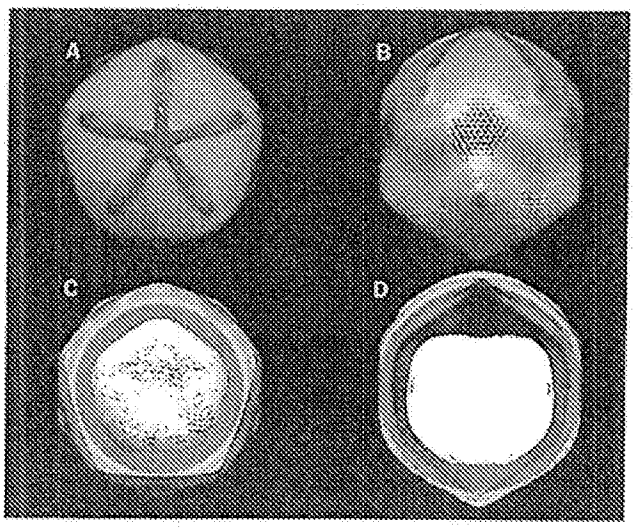
The team also knocked out a gene that directs the conversion of vanillin to an undesirable form. The researchers say they were pleased with the yields: Fission yeast made 65 milligrams per liter of liquid in the flasks, baker's yeast 45 mg/l.

To further increase the yeast yield of vanillin, the researchers added an additional gene that encodes for an enzyme that converts the straight vanillin into a form with a sugar attached, vanillin beta-D-glucoside. This form isn't toxic, says Møller, allowing the yeast to hold more of the compound. Both the straight and sugar-laden vanillin could be used in foods and perfumes.

While synthetic vanillin doesn't offer the rich flavors of true vanilla, the artificial form has its place, says Daphna Havkin-Frenkel, director of research and development at Bakto Flavors in Rutgers, N.J.

A good look at mimi

Scientists have zoomed in on mimivirus, the enormous virus with the delicate name that has perplexed researchers since its discovery in 1992. Its size (its diameter is more than 10 times that of the virus that causes the common cold) and its hodgepodge of genetic and structural traits blur the line of what is alive, says Michael Rossmann of Purdue University in West Lafayette, Ind. Rossmann and an international team report the results of their reconnaissance online April 28 in *PLoS Biology*. Cryo-electron microscopy images reveal the details of a starfish-shaped structure (A, B) that covers an opening in the virus coat through which DNA might be expelled when the virus infects a host. The DNA is enveloped in a membrane, seen in gray in reconstructed renderings (C, D). The new work may help scientists understand if and how the virus could cause disease. —*Rachel Ehrenberg*



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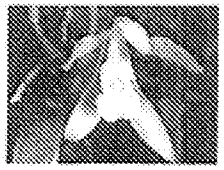
YEAST BRED TO BEAR ARTIFICIAL VANILLA

Researchers have co-opted fungi to produce the flavor more efficiently

by Rachel Ehrenberg
May 23rd, 2009; Vol.175 #11 (p. 9)



Enlarge

True vanilla is an extract from the seed-bearing pods of *Vanilla planifolia* (above) or *Vanilla tahitensis*.

National Park Service Photo

A jug of wine, a loaf of bread and now, vanilla.

Yeast has long been pressed into service for making food and drink, and now scientists have recruited the fungus for a loftier flavor: vanillin, vanilla's dominant compound. Scientists report in an upcoming *Applied and Environmental Microbiology* that they have engineered strains of beer and baker's yeast to produce vanillin from glucose, a greener and cheaper route than previous methods.

"This is absolutely beautiful work," says John Rosazza, a medicinal and natural products chemist at the University of Iowa in Iowa City. There is a huge market for vanillin, Rosazza says.

Vanillin is the dominant compound of the hundreds that are found in vanilla — an extract from the seed-bearing pods, called beans, of two orchids, *Vanilla planifolia* and *Vanilla tahitensis*. But real vanilla beans are precious, rare and costly. Today, less than a percent of the vanillin sold each year is derived from the orchids. The majority of vanillin is synthesized in chemistry labs, and typically made from lignin, a constituent of wood left over from the paper-making industry, or guaiacol, which is derived from wood creosote.

Enlarge

Scientists have engineered two species of yeast to make vanillin (above), the dominant flavor compound in vanilla.

Scientists have also used microorganisms in a multistep process to make vanillin from two plant compounds, ferulic acid and eugenol. But these precursors are expensive and the process involves environmentally unfriendly chemicals, says Jergen Hansen of Evolva Biotech's Copenhagen office. Also, vanillin itself is toxic to many microorganisms, complicating matters.

Now Hansen, Birger Lindberg Moller of the University of Copenhagen in Denmark and colleagues created a chemistry lab of their own within two different species of yeast: *Schizosaccharomyces pombe*, also known as fission or beer yeast, and baker's or brewer's yeast, *Saccharomyces cerevisiae*. Instead of using the typical expensive starting material, the team turned to glucose, a cheap and available sugar. To make the yeast convert the glucose to vanillin, the researchers added genes that encode for specific enzymes that spur the reactions. These genes included one from the dung mold *Podospora pauciseta*, two bacterial genes and a human gene.

The team also knocked out the gene that directs the conversion of vanillin to an undesirable form. The researchers report that they were pleased with the yields: the beer yeast made 65 milligrams per liter, the baker's yeast 45 mg/l.

To further increase the yeast yield of vanillin, the researchers then added an additional gene that encodes for a plant enzyme that converts the straight vanillin into a form with a sugar attached, vanillin beta-D-glucoside. This form isn't toxic at all, says Moller, allowing the yeast to hold much more the compound. And because the added sugar is easily broken down in the mouth or on the skin, both the straight and sugar-laden vanillin could be used in foods and perfumes.

Vanillin may also find its way into pain-relieving drugs, Moller says. Vanillin is one of the molecules in the biochemical pathway that leads to capsaicin, the compound that gives chili peppers their heat and is under investigation as a pain reliever.

"Somehow all people like vanilla," he says. "Why? Is it the immediate taste? Does it hide some pain we're not aware is there?"

While synthetic vanillin doesn't offer the rich flavors of true vanilla, the artificial form has its place, says Daphna Havkin-Frenkel, director of research and development at Bakto Flavors in Rutgers, N.J.

If you seek real vanilla, though, read your labels carefully, she says. Calling synthetic vanilla real vanilla "is almost a political problem," she says. "People are very passionate about vanilla."